AD_____

Award Number: W81XWH-08-1-0305

TITLE: Specific PET Imaging Probes for Early Detection of Prostate Cancer Metastases

PRINCIPAL INVESTIGATOR: Xiankai Sun

CONTRACTING ORGANIZATION:
University of Texas
Dallas, Texas 75390

REPORT DATE: May 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☒ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
31/05/2009	Annual	1 MAY 2008-30 APR 2009

4. TITLE AND SUBTITLE
Specific PET Imaging Probes for Early Detection of Prostate Cancer Metastases

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0305

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Xiankai Sun

Email: xiankai.sun@utsouthwestern.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas

Dallas, Texas 75390

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
N/A

14. ABSTRACT: Polyarginines are a group of small peptides that have been used as drug delivery vehicles due to their capability of penetrating cell membranes. In one of our studies using such a peptide to deliver a therapeutic moiety to various prostate cancer cell lines, we surprisingly discovered that the peptide had remarkably high preference to prostate tissues. This specificity, which has not been reported before, prompted us to exploit this group of peptides for the early detection of prostate tumor metastases. Promisingly, in our preliminary studies, the peptide labeled with ^{64}Cu can clearly reveal metastases in a tumor-bearing animal model. In the first year of this project, we have shown that glycosaminoglycans play an important role in the uptake of the peptide in four prostate cancer cell lines. Among the inhibitors tested, dextran sulfate, protamin sulfate and pentosan sulfate are highly potent. Our biodistribution studies indicated that the preference uptake was specific to the peptide length of polyarginines. Further we have accomplished the synthesis of the proposed multifunctional chelators and the construction of their multivalent peptide conjugates. The peptide constructs were efficiently labeled with ^{64}Cu under mild conditions with 30-min. Preliminary in vitro binding assay demonstrated the desired multivalent effect rendered by the designed scaffold, and the radiolabeled conjugates showed high in vitro and in vivo stability.

15. SUBJECT TERMS - None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Xiankai Sun
unclassified	unclassified	unclassified	unlimited	16	19b. TELEPHONE NUMBER *(include area code)* 214-645-5978

Table of Contents

Introduction

The ultimate goal of this project is to develop specific PET imaging probes for early detection of distal metastases of prostate cancer. Based on one of our studies using a polyarginine (NH_2GR11) to deliver a therapeutic moiety to various prostate cancer cell lines, we hypothesize a new group of prostate-specific peptides could be developed as novel PET imaging probes using NH_2GR11 as lead compound for the detection of multi-foci extraprostatic spread of prostate cancer. Three specific aims are arranged to achieve the goals of this proposal: **(I)** Determine the mechanism of the prostate-specific uptake exhibited by NH_2GR11; (II) Design and synthesize novel BFC-peptide conjugates in order to achieve desired in vivo stability, pharmacokinetics, and enhanced prostate-specific binding affinity; and (III) Establish radiochemical protocols to label peptide conjugates with ^{64}Cu, and perform in vitro/*in vivo* evaluations of the potential prostate cancer-specific imaging agents.

Body

In the statement of work, the focus of our 1st year work was on part of Objectives I, II, and III. Specifically,

Months 0 – 12:
Task 1: Dissecting mechanism of R11 uptake via interacting with "specific" GAG (Objective I)
Task 2: Synthesis of L-, D-, or mixed L/D polyargines (Objective II)
Task 3: In vitro and in vivo evaluation of the FITC-tagged polyarginines (Objective II)

Months 6 – 24:
Task 4: Prepare p-SCN-Bn-CB-TE2A and the multivalent scaffolds (CB-TE2O-(PEG-COOH)$_2$ or CB-TE2A-(PEG-COOH)$_2$) and their peptide conjugates (Objective II)

Months 6 – 12:

Task 5: Establishing radiochemical protocols to label the peptide conjugates with ^{64}Cu (Objective III)

Research Progress in the 1st year

Task 1: Dissecting mechanism of R11 uptake via interacting with "specific" GAG (completed)
Task 2: Synthesis of L-, D-, or mixed L/D polyargines
Task 3 (completed): In vitro and in vivo evaluation of the FITC-tagged polyarginines (completed)
Task 4: Prepare p-SCN-Bn-CB-TE2A and the multivalent scaffolds (CB-TE2O-(PEG-COOH)$_2$ or CB-TE2A-(PEG-COOH)$_2$) and their peptide conjugates (Completed)
Task 5: Establishing radiochemical protocols to label the peptide conjugates with ^{64}Cu (Completed)

Task 1:
Characterization of polyarginine (i.e., FITC-R9, FITC-R11 and FITC-R13) uptake in various prostate cancer cell lines under the influence of different Glycosaminoglycans (GAGs)
As shown in **Figure 1**, the results can be summarized as follows:

1. The uptake of polyarginine appears to be temperature-independent.

2. Several inhibitors (EIPA for macropinocytosis and Toxin B for Rac), EIPA (5-(*N*-ethyl-*N*-isopropyl) amiloride) can block all three polyarginines uptake very efficiently but not Toxin B. Other inhibitors for glycoprotein and proteoglycan synthesis are not effective.
3. In a competition study (add FITC-R11 and GAG simultaneously into cell), dextran sulfate, protamin sulfate and pentosan sulfate are more potent than heparan sulfate and chondroitin sulfates (A, B, C) to inhibit FITC-R11 uptake.
4. In a pre-incubation study (incubate GAG 10, 30, 120, 360 min then wash prior to FITC-R11 addition), both dextran sulfate and pentosan sulfate remain active but not protamin sulfate. In addition, heparan sulfate becomes potent.
5. In an experiment, cells were incubated with FITC-R11 and different carbohydrates such as glucose, dextran, fructose, mannose, galactose. It appears that all the carbohydrates can inhibit ca. 50% of FITC-R11 uptake.

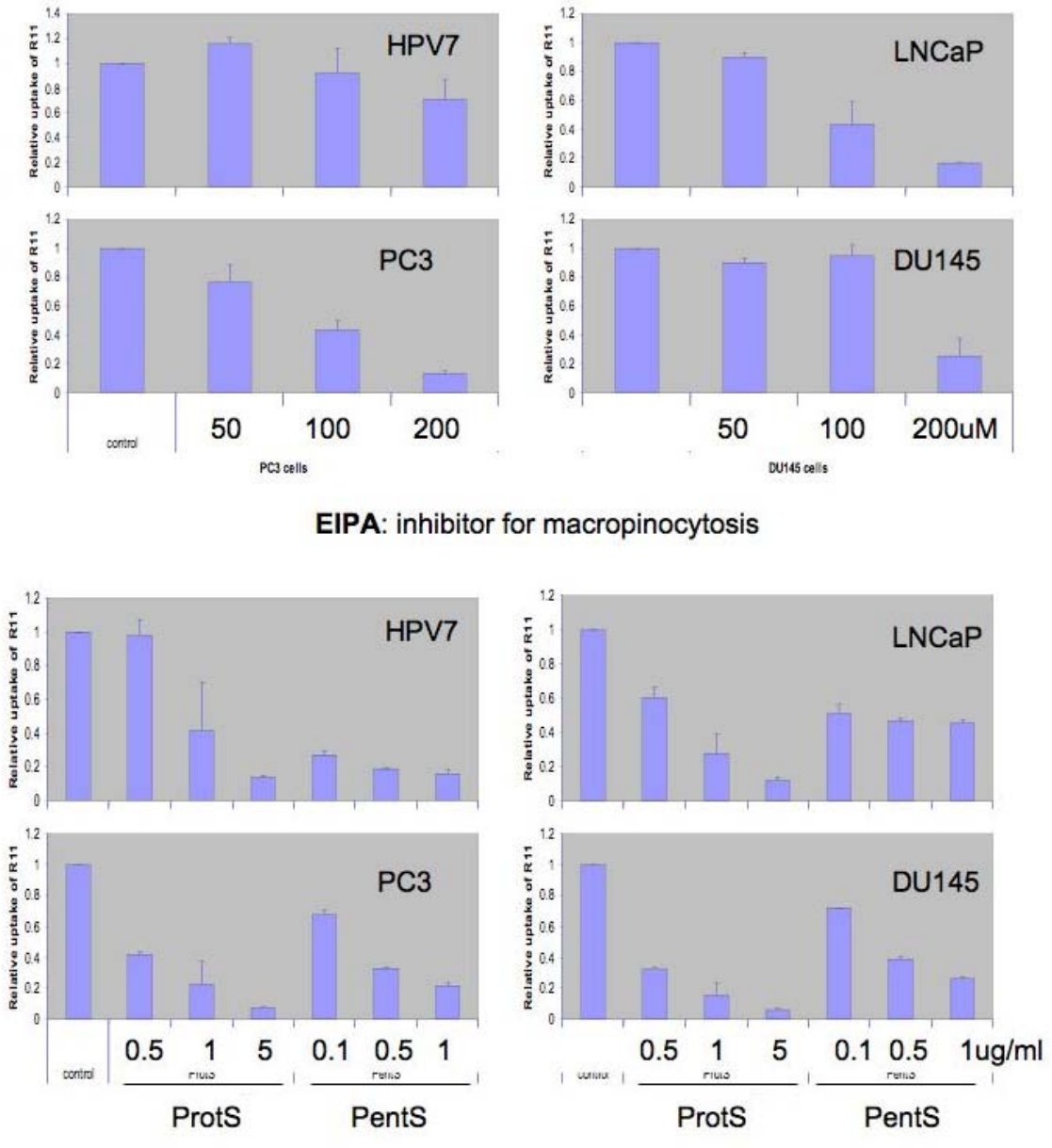


Figure 1. Inhibition of FITC-R11 uptake in various prostate cell lines

As such we have planed to identify the binding protein(s) of FITC-R11 using gel electrophoresis and affinity column purification. For gel electrophoresis, we will use: 1) 4 °C as an incubation condition; 2) 5 μM of FITC-R11 (or lower) plus 10-min incubation time as a start; 3) 20 μg total cell lysate (cytosol or cell membrane extract) as a start; and 4) pentosan sulfate and excess unlabeled-R11 as the competitors.

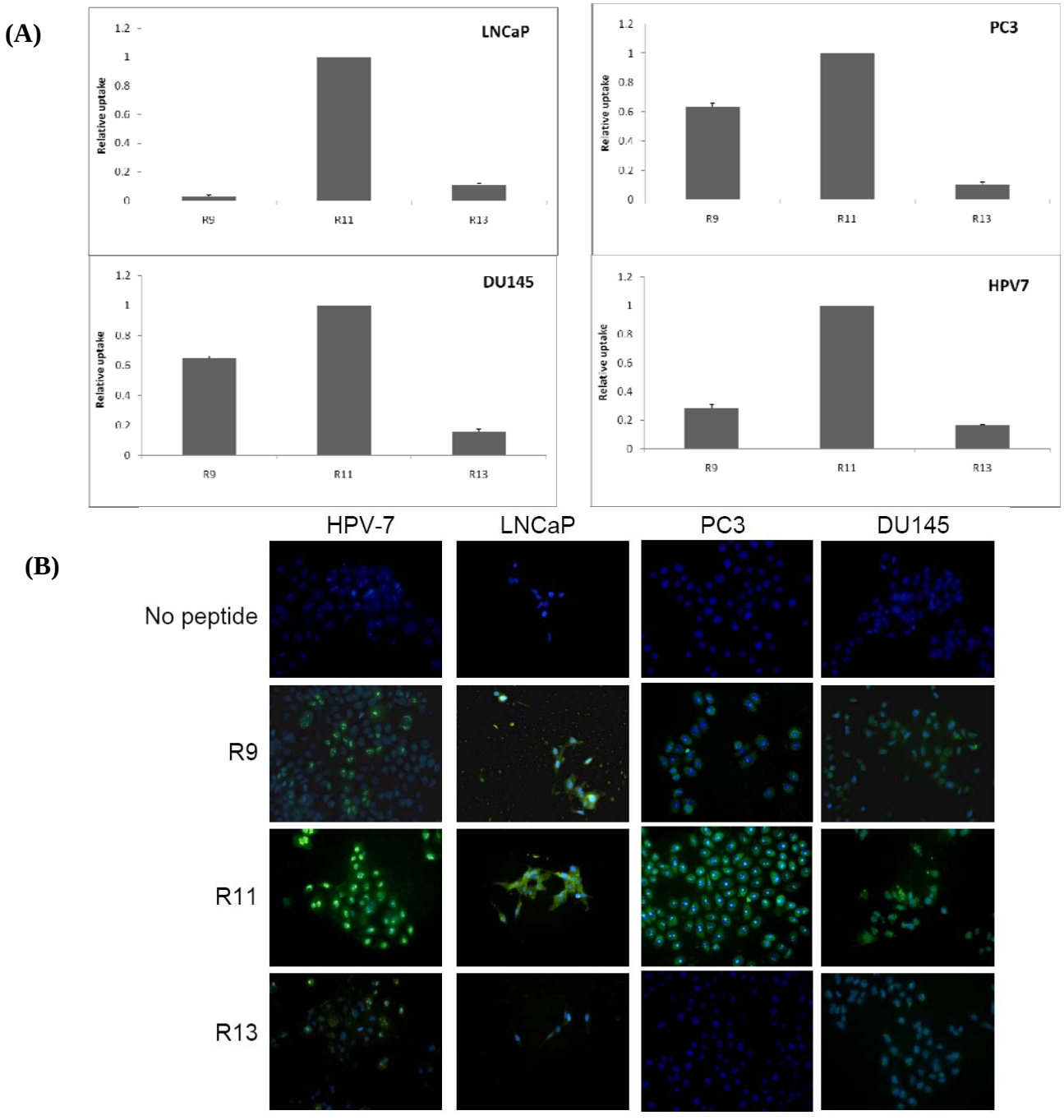


Figure 2. Uptake of R9, R11 and R13 by prostate cancer cells (LNCaP, PC3, DU145 and HPV7). *A*, different concentrations of each R9, R11 and R13 were incubated with cells for 30 min before cell harvesting. Relative FITC intensity was determined by normalizing fluorescence intensity with its protein content and presented as relative level compared to uptake level of R11. All the experiments were duplicated in triplicates. *B*, cells were incubated with 5 μmol/L of the indicated peptide for 30 min. After fixation, cells were counterstained with DAPI. The cellular distribution of each peptide was visualized with fluorescence microscope.

Task 2: Based on our in vitro and in vivo evaluation, NH_2GR11 peptide is highly stable in the form of either FITC-R11 or DOTA-R11. Out to 48 h post injection (via the tail vein), the peptide remained > 80% intact. The D- or mixed D/L forms of the peptide was proposed in case we would have the stability issue with the L-homopolymers. Given the in vivo stability is no longer a concern, we have decided to skip this task.

Task 3:

In vitro cell uptake and subcellular localization: The uptake of FITC-R9, FITC-R11 and FITC-R13 in four different prostate cancer cells was examined. As shown in Figure 2, FITC-R11 exhibited the highest uptake in all the four cell lines, indicating the efficiency of uptake was specific to the peptide length. In addition, we found out that FITC-R11 could be more efficiently internalized than any of the other peptides. Most of the internalized peptide molecules were localized in the cytosol, with few nuclear staining seen in PZ-HPV-7 and PC3 cells.

Biodistribution of FITC-R11: The biodistribution data of FITC-R11 in normal Balb/c mice are presented in Figure 3. At 24 h post-injection (p.i.), FITC-R11 showed the highest uptake in the prostate, seminal vesicle (SV), coagulation gland (CG) among the three polyarginines. In other organs all the three CPPs showed similar and low uptake. In nude mice bearing PC-3 tumors, FITC-R11 also exhibited high tumor uptake (Figure 4) with a tumor/muscle ratio of ~ 4. The biodistribution profiles of the peptides in normal and tumor-bearing mice were similar.

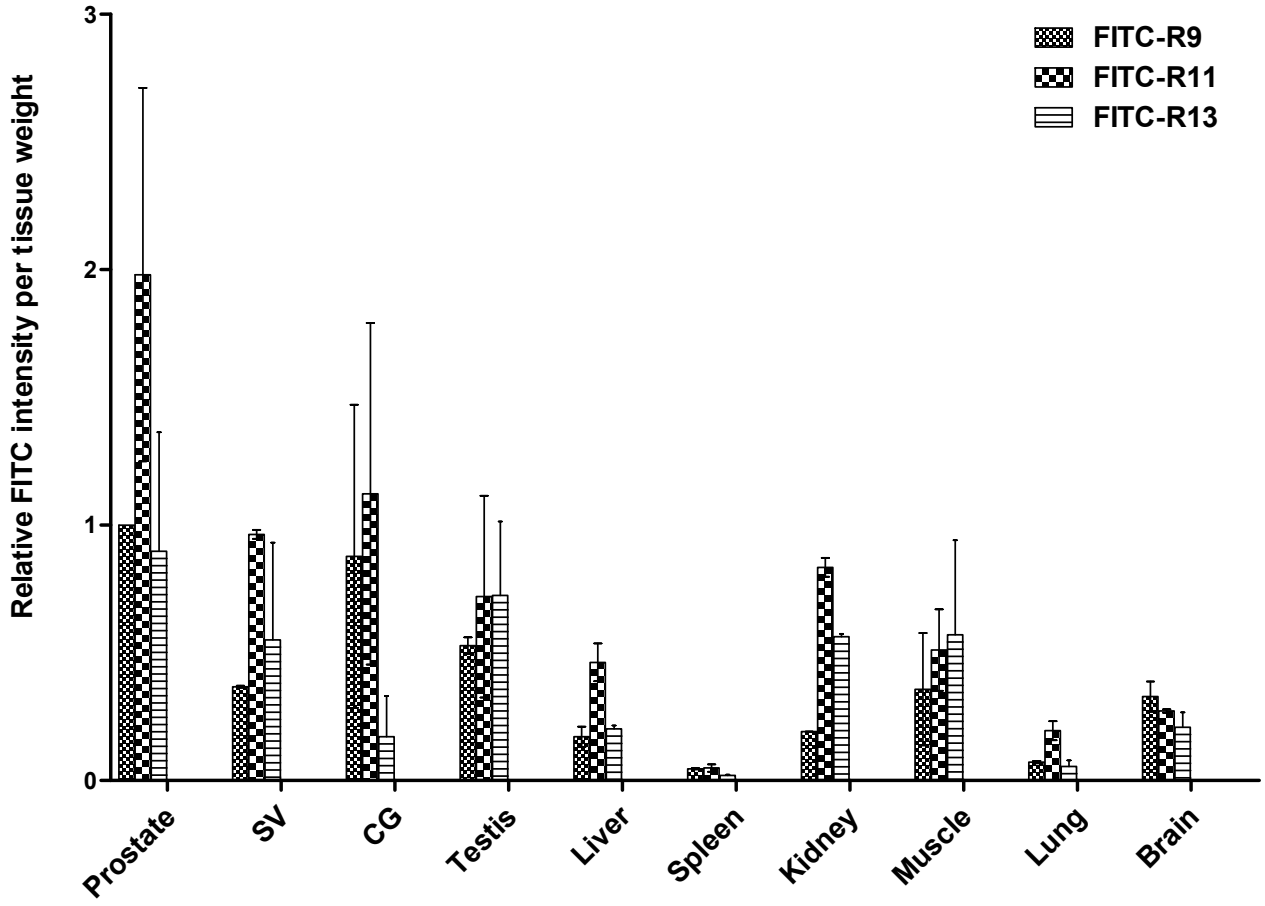


Figure 2. Biodistribution of FITC-R11 in normal male nu/nu nude mice at 24 h p.i. (n = 4). SV: seminal vesicle, CG: coagulation gland.

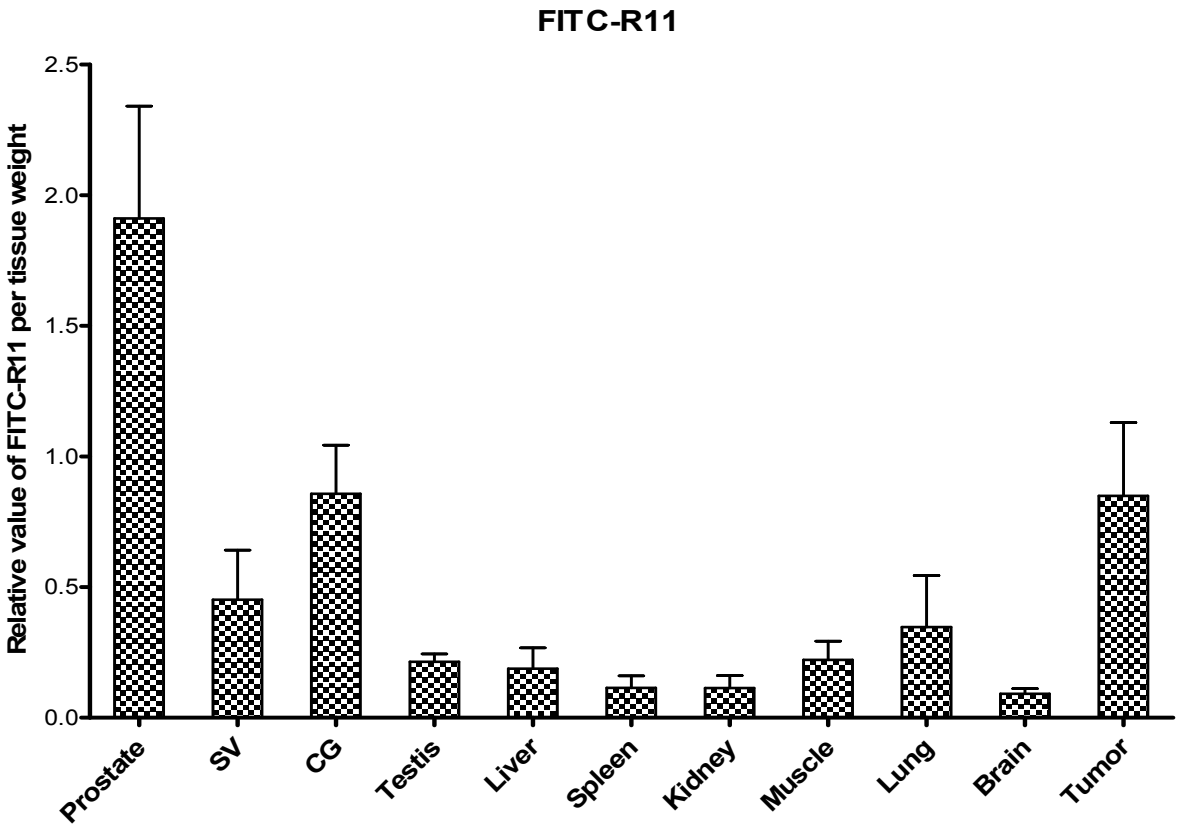


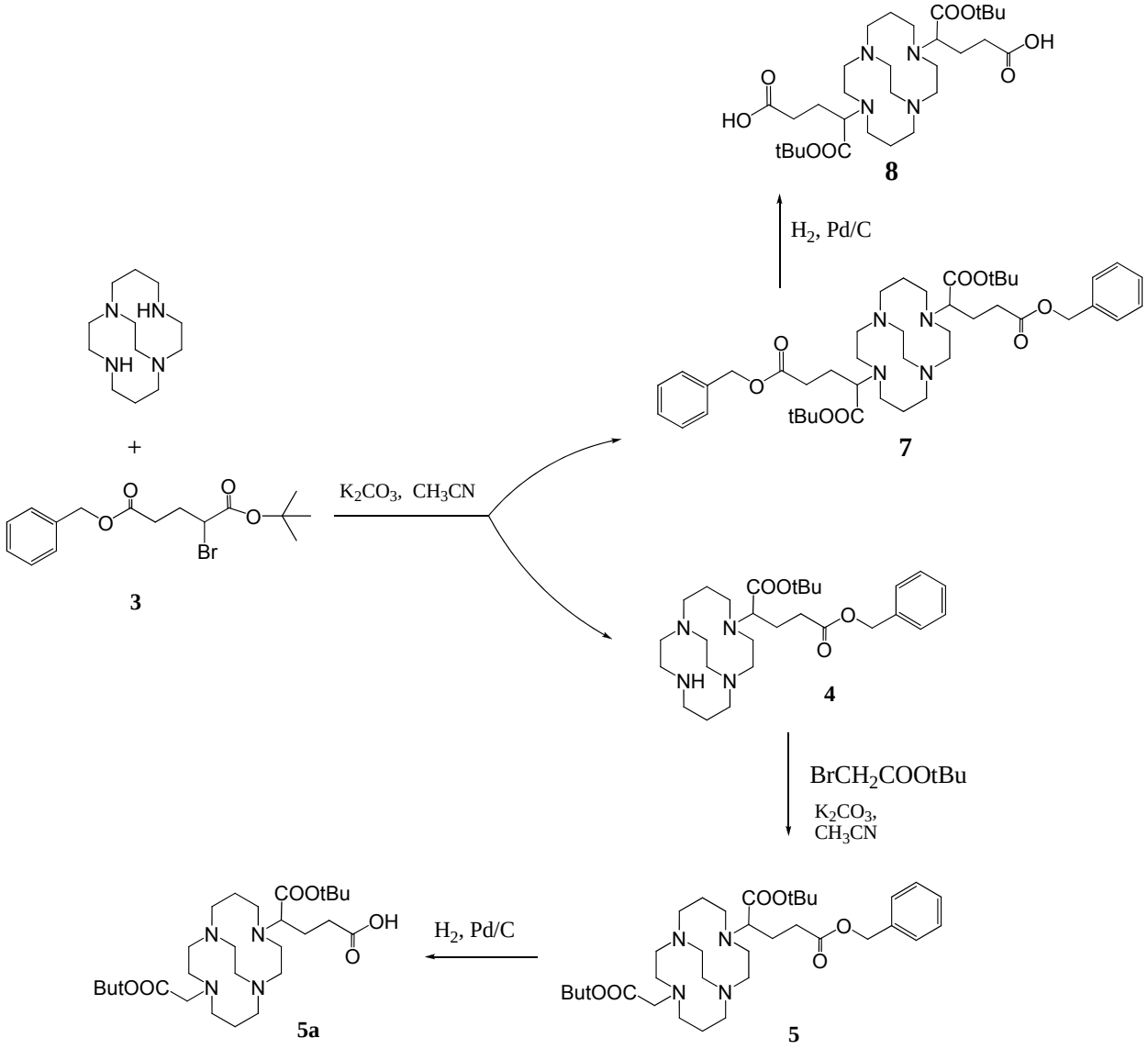
Figure 3. Biodistribution of FITC-R11 in PC-3 tumor-bearing mice at 24 h p.i. (n = 4).

Polyarginines as a group of cell peameation peptides (CPPs) have shown efficient cell internalization. Hydrogen-bond formation of the guanidino moiety in arginine with phosphates, sulfates, and carboxylates on cellular components has been considered to be crucial to the internalization efficacy (*1*). Polyarginines are highly cationic and strongly adsorbed on membrane surfaces, and are often difficult to be removed completely from the cell surface where they have a particular affinity. Several delivery mechanisms including endocytic and non-endocytic uptake have been proposed to explain how polyarginines traverse cellular membranes (*2*). Multiple internalization pathways are possible in simultaneous actions and their contributions are dependent on a numbers of factors. Under defined conditions endocytic pathways, including macropinocytosis, are the predominant internalization routes for polyarginines (*3*). However, diffuse cytosolic labeling by polyarginines can be typically observed under the conditions where endocytosis is inhibited. Inhibition of cellular uptake by endocytosis may in fact lead to the accumulation of the peptides on cell surface to yield diffuse cytosolic labeling (*1*). Diffuse, direct, and non-endocytic mechanism become predominant at higher peptide concentrations (*4*). It was also reported that no special receptors existed for polyarginines and there were no essential selectivity of polyarginines toward different organs and tissues (*5, 6*). However, in our *in vivo* evaluation of tissue distribution of FITC-tagged polyarginines in nude mice, FITC-R11 exhibited a significant preference to accumulate in the prostate, while its uptake in other tissues was much lower. Among R9, R11, and R13, R11 showed the highest uptake in all four kinds of prostate cancer cells. R11 also had significantly higher uptake than other CPPs (TAT, PENE, KALA and K11) in prostate cancer cells (LNCaP, C4-2, LAPC4 and PC3) from our previous study (*7*). These observations clearly demonstrate that R11 has tissue specificity toward the prostate in addition to its cell membrane translocation feature, although the mechanism is not so well understood.

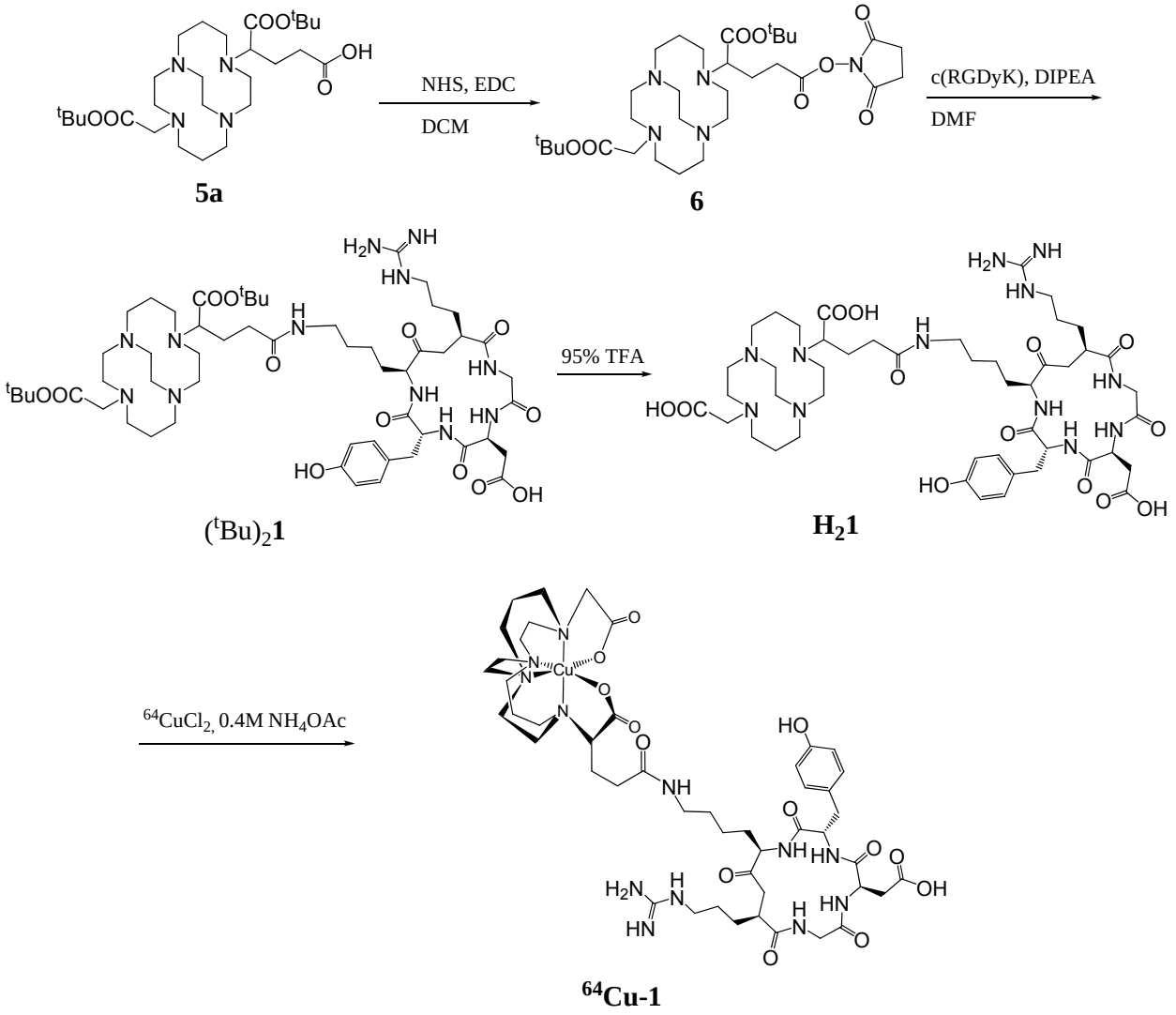
Task 4: Prepare p-SCN-Bn-CB-TE2A and the multivalent scaffolds (CB-TE2O-(PEG-COOH)$_2$ or CB-TE2A-(PEG-COOH)$_2$) and their peptide conjugates.
Task 5: Establishing radiochemical protocols to label the peptide conjugates with ^{64}Cu.

Since NH$_2$GR11 is still under evaluation, we chose a well established peptide, c(RGDyK), which targets the $\alpha_v\beta_3$ integrin (an angiogenesis biomarker), for the construction of our proposed peptide conjugates. The chemical and radiochemical procedures are outlined in Schemes 1 – 3:

Scheme 1

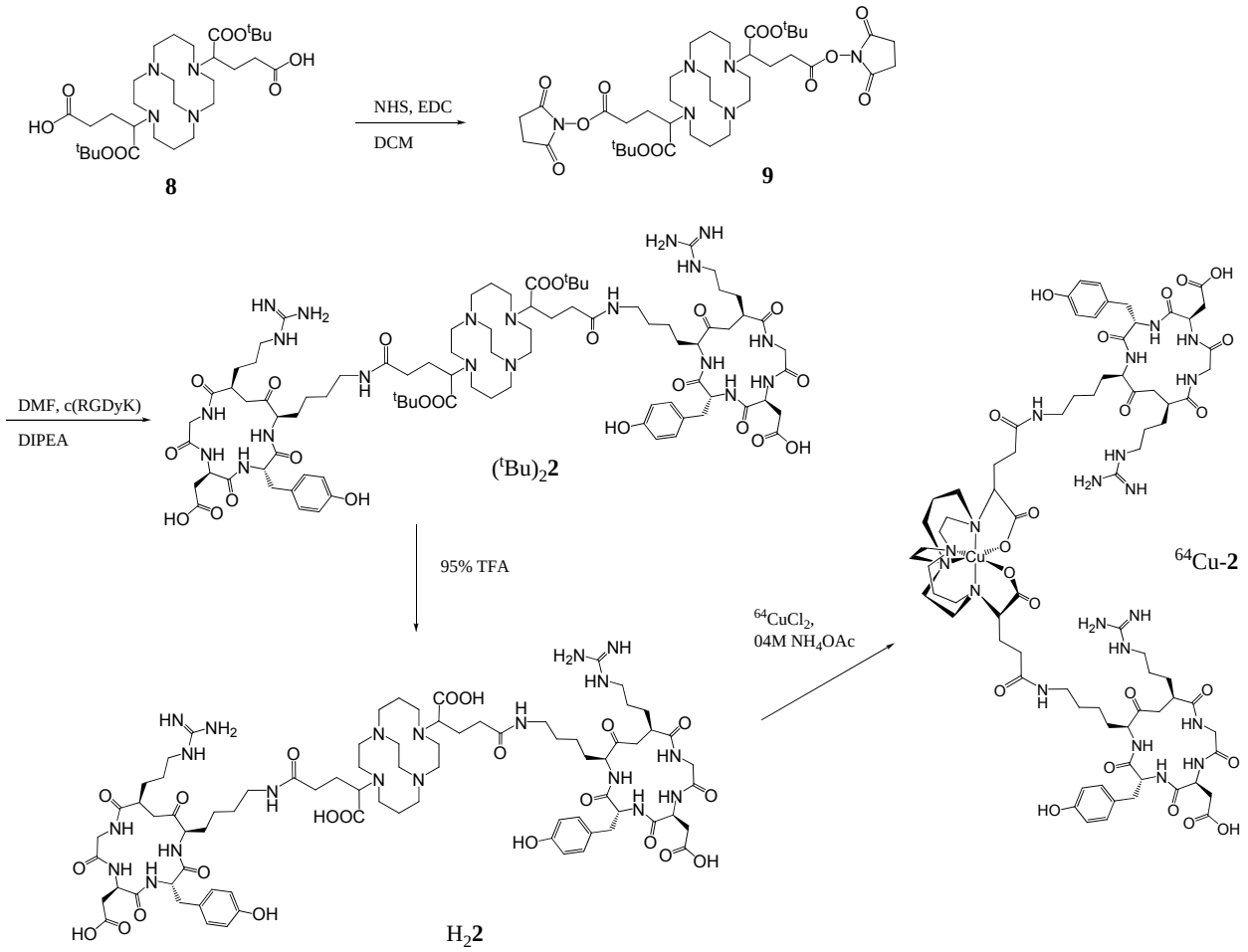


Scheme 2



5a **6** $(^{t}Bu)_2$**1** $\mathbf{H_2}$**1** ^{64}**Cu-1**

Scheme 3



5-benzyl 1-tert-butyl 2,2'-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyl)dipentane-dioate (4; Scheme 1). To a suspension of cross-bridge cyclam (230 mg, 1.03mmol) and K_2CO_3 (0.57 g, 4.1 mmol) in 5 mL of anhydrous acetonitrile at 0°C, compound **3** (384 mg, 1.08 mmol) in 2 mL of anhydrous MeCN was added dropwise. After the addition, the reaction was maintained at 0°C for 5 h, and then allowed to proceed at room temperature (r. t.) for 3 days. The solids were removed by filtration and washed with chloroform (2 × 20 mL). The combined filtrate was concentrated under vacuum and purified by column chromatography (silica gel, 60-230 mesh) using 10:1 $CHCl_3$/MeOH to 9:1 EtOAc/isopropyl amine (R_f = 0.43) for elution. Compound **4** was obtained as a sticky oil (286 mg; Yield: 55%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.42 (s, 9H), 1.78-1.95 (m, 1H), 1.98-2.13 (m, 1H), 2.14-2.21 (m, 1H), 2.34-3.25 (m, 26H), 5.09 (s, 2H), 7.32 (s, 5H); ^{13}C NMR ($CDCl_3$,100 MHz) δ 25.43, 26.17, 27.48, 28.56, 31.73, 48.92, 49.16, 49.38, 52.04, 53.94, 54.11, 57.90, 59.15, 60.40, 62.04, 66.50, 81.24, 128.45, 128.78, 136.16, 172.59, 173.27. MALDI-TOF/MS: 503.71 [M + H^+].

5-benzyl 1-tert-butyl 2-(11-(2-tert-butoxy-2-oxoethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexa-decan-4-yl)pentanedioate (5; Scheme 1). To 2 mL of **4** (100 mg, 0.20 mmol) in acetonitrile, K_2CO_3 (120mg, 0.87 mmol) was added followed by methyl chloroacetate (39 mg, 0.20 mmol) in 2 mL of acetonitrile. The resulting mixture was stirred at r. t. for 2 days. After removal of the solids by filtration and evaporation of

the solvent, the residue was redissolved in chloroform and washed thoroughly with water. The organic layer was dried over sodium sulfate. Removal of the solvent under vacuum afforded the crude product as a brown oil, which was purified by column chromatography (silica gel, 60-230 mesh) using 10:1 $CHCl_3$/MeOH to 9:1 EtOAc/isopropyl amine (R_f = 0.60) for elution. Compound **5** was obtained as a pale yellow sticky oil (56 mg; Yield: 92%): ^{1}H NMR ($CDCl_3$, 500 MHz)δ 1.46 (s, 18H), 1.73-3.20 (m, 29H), 3.58 (m, 1H), 3.90 (m, 1H), 5.12 (s, 2H), 7.36 (s, 5H); MALDI-TOF/MS: 617.12 [M + H^+].

Compound (^{t}Bu)$_2$1 (Scheme 2). To a solution of **5** (100 mg, 0.16 mmol) in 5 mL of mixture solvent THF/H_2O (1:1) was added portion wise 10 mg of 10% Pd/C. The suspension was shaken in a hydrogenator (Parr, Moline, Illionis) at r. t. for 12 h under an H_2 atmosphere (60 psi). After removal of the solids, evaporation of solvent and lyophilize to remove the remaining water, compound **5a** was obtained as a white foam in nearly quantitative yield. Then a mixture of compound **5a** (100mg, 0.19 mmol), *N*-hydroxysuccinimide (43mg, 0.38 mmol) and EDC·HCl (73 mg, 0.38 μmol) in 1 mL of dry acetonitrile was stirred under N_2 for 24 hours. After removal of the solvent under reduced pressure, the residue was dissolved in chloroform (1 mL) and was washed 3 times (3 × 2 mL) by water promptly. Upon the removal of chloroform under vacuum, the solution was lyophilized to afford a pale yellow solid **6**. Compound **6** was used directly for the next reaction without further purification. A cyclic RGD peptide [c(RGDyK)] (10 mg, 16 μmol) was then mixed with **6** (12 mg, 19 μmol) in 500 μL of anhydrous DMF, to which 30 μL of N, N-diisopropylethylamine (DIPEA) was added (*ca.* 10 equivalent to RGD). The mixture was stirred at room temperature for two days. After removal of the solvent, the crude product was purified by semi-preparative reverse-phase HPLC. The collected fractions from multiple runs were pooled and lyophilized to afford (^{t}Bu)$_2$**1** as a white foam (5.0 mg; Yield: 29%). MALDI-TOF/MS: 1127.88 [M+H^+].

Compound $H_2$1 (Scheme 2). Compound (^{t}Bu)$_2$**1** (5 mg, 4.4 μmol) was dissolved in 95% of TFA and stirred at room temperature for 12 h. After evaporation of the solvent, the residue was purified by semi-preparative reverse-phase HPLC. The collected fractions were from multiple runs were pooled and lyophilized to afford a white solid at quantitative yield. MALDI-TOF/MS: 1015.03 [M+H^+].

5-Benzyl 1-tert-butyl 2-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecan-4-yl)pentanedioate (7; Scheme 1). To a suspension of cross-bridge cyclam (180 mg, 0.80 mmol) and K_2CO_3 (1.2 g, 8.68 mmol) in 5 mL of anhydrous of acetonitrile was added compound **3** (630 mg, 1.76 mmol) in 2 mL of anhydrous acetonitrile was added. The reaction was stirred at r. t. for 24 h and then the temperature was elevated to and kept at 50 °C for 24 h. The solids were removed by filtration and washed with chloroform (2 × 20 mL). The combined filtrate was concentrated under vacuum and purified by column chromatography (silica gel, .60-230 mesh) using 10:1 $CHCl_3$/MeOH to 9:1 EtOAc/isopropyl amine (R_f = 0.84) for elution. Compound **7** was obtained as a sticky oil (390.6 mg; Yield: 45%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.44 (s, 18H), 1.79-1.90 (m, 2H), 1.92-2.80 (m, 2H), 2.23-3.20 (m, 28H), 3.59-3.68 (m, 1H), 3.72-3.85 (m, 1H), 5.11 (s, 4H), 7.34 (s, 10H); ^{13}C NMR ($CDCl_3$,100 MHz) δ 25.29, 28.38, 30.91, 50.32 (br), 51.67 (br), 53.34 (br), 55.89 (br), 62.43, 66.75, 82.69, 128.49, 128.63, 128.85, 135.99, 171.22, 172.65. MALDI-TOF/MS: 779.60 [M+H^+]. Elemental Anal for **7**·0.25CH_3CN·0.1$CHCl_3$: C 66.86%; H 8.41%; N 7.43%. Found: C 66.93%; H 8.45%; N 7.48%.

4,4'-(1,4,8,11-tetraazabicyclo[6.6.2]hexadecane-4,11-diyl)bis(5-tert-butoxy-5-oxopentanoic acid) (8; Scheme 1). To a solution of **7** (100 mg, 0.13 mmol) in 5 mL of THF/H_2O (2:1) was added portionwise 10 mg of 10% Pd/C. The suspension was shaken at r. t. for 16 h under a hydrogen atmosphere (60 psi) in a hydrogenator. After removal of the solids, evaporation of the solvents under vacuum afforded compound **8** as a white solid in quantitative yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.40 (s, 18H), 1.56-2.00 (m, 8H), 2.34-2.33 (m, 4H), 3.30-2.73 (m, 22H) ; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 24.58, 27.36, 27.65, 31.95, 50.27

(br), 51.52 (br), 54.21(br), 62.62, 83.94, 173.72, 177.99; MALDI-TOF/MS: 599.60 [M+H$^+$]. Elemental Anal for K_2**8**·0.5THF·0.5H_2O: C 53.38%; H 7.98%; N 7.78%. Found: C 53.48%; H 8.11%; N 7.94%.

Compound (^{t}Bu)$_2$2 (Scheme 3). A mixture of compound **8** (10.0 mg, 16.7 μmol), *N*-hydroxysuccinimide (7.6 mg , 66.8 μmol) and EDC·HCl (12.8 mg, 66.8 μmol) in 500 μL of dry acetonitrile was stirred under N_2 for 24 h. After removal of the solvent under reduced pressure, the residue was redissolved in chloroform (1 mL) and then washed 3 times with water (3 × 2 mL). After evaporation of chloroform, the residue was dried by a lyophilizer to yield a pale yellow solid. The NHS-activated ester (compound **9**; Scheme S3) was used directly for the RGD conjugation without further purification. Cyclic RGD peptide [c(RGDyK)] (10 mg, 16 μmol) was mixed with **9** (2.4 mg, 4 μmol) in 200 μL of anhydrous DMF, to which 30 μL of N, N-diisopropylethylamine (DIPEA) was added (*ca.* 10 equivalent to RGD). The mixture was stirred at r. t. for 24 h under a nitrogen atmosphere. After evaporation of the solvent under vacuum, the crude product was purified by semi-preparative reverse-phase HPLC. The collected fractions of multiple runs were pooled and lyophilized to afford 2.2 mg of (^{t}Bu)$_2$**2** as a white powder (30%). MALDI-TOF/MS: 1800.12 [M+H$^+$].

Compound $H_2$2 (Scheme 3). Compound (^{t}Bu)$_2$**2** (2 mg, 1.1 μmol) was dissolved in 95% of TFA and stirred at r. t. for 12 h. After evaporation of the solvent, the residue was purified by semi-preparative reverse-phase HPLC. The collected fractions of multiple runs were pooled and lyophilized to afford a white solid (H_2**2**) in quantitative yield. MALDI-TOF/MS: 1688.73 [M+H$^+$].

Radiolabeling of $H_2$1 or $H_2$2 with ^{64}Cu: To a 1.5 mL vial containing 5 μg of H_2**1** or H_2**2** in 200 μL of 0.4 M NH_4OAc (pH = 6.5) solution, 2 – 3 mCi of $^{64}CuCl_2$ in 0.1 M HCl was added. The reaction mixture was shaken and incubated at 75ºC for 0.5 h. Then, 5 μL of 5 mM diethylenetriaminepentaacetic acid (DTPA) was added to the reaction mixture, which was allowed to incubate for another 5 min (DTPA was used to remove non-specifically bound or free ^{64}Cu from the ^{64}Cu-labeled **1** or **2**). The purification of ^{64}Cu-**1** or ^{64}Cu-**2** was carried out by passing the mixture through a Sep-Pak C-18 plus cartridge. After thorough rinsing the cartridge two times with water, the ^{64}Cu-labeled product was eluted out by pure ethanol. The product was firstly analyzed by radio-TLC and then by radio-HPLC to determine the radiochemical purity of the product.

Serum Stability: An aliquot (*ca.* 40 μCi) of ^{64}Cu-**1** or ^{64}Cu-**2** was added into each of 12 vials containing 100 μL of rat serum. The vials were incubated at 37 ºC in a water bath. At each time point (1, 2, 4 and 24 h), 420 μL of ethanol was added to three of the vials to precipitate the serum proteins. After high speed (14,000 rpm) centrifugation for 5 min, the supernatant was removed and then the pellet was resuspended with 240 μL of 80% ethanol. The suspension was centrifuged again and the supernatant was collected. The supernatants were pooled and then analyzed by radio-HPLC. The dislodged ^{64}Cu from ^{64}Cu-**1** or ^{64}Cu-**2** if any is assumed to be associated with the serum proteins.

Cell Integrin Receptor-Binding Assay: The $\alpha_\upsilon\beta_3$ integrin-binding affinities of ^{64}Cu-**1** and ^{64}Cu-**2**. were determined by a competitive cell-binding assay using ^{125}I-echistatin (PerkinElmer) as the $\alpha_\upsilon\beta_3$-specific radioligand. The experiments were performed on U87MG human glioblastoma cells by a previously reported method (*8, 9*). Briefly, U87MG cells were grown in Dulbecco's modified Eagle medium (DMEM, low glucose) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37ºC with 5% CO_2. Suspended U87MG cells in binding buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1% bovine serum albumin) were seeded on multi-screen DV plates (Millipore) with 2 × 10^5 cells per well and then incubated with ^{125}I-echistatin (10,000 cpm/well) in the presence of increasing concentrations (0 – 5,000 nM) of c(RGDyK) peptide conjugates for 2 h. The final volume in each well was maintained at 200 μL. At the end of incubation, the unbound ^{125}I-echistatin was removed by filtration and

then three times of rinsing with cold binding buffer. The filters were collected and the radioactivity was measured using a γ-counter. The best-fit IC_{50} values (inhibitory concentration where 50% of the ^{125}I-echistatin bound on U87MG cells are displaced) of c(RGDyK), H_2**1**, and H_2**2** were calculated by fitting the data with nonlinear regression using GraphPad Prism (GraphPad Software, Inc.). Experiments were duplicated with quintuplicate samples. The results are shown in Figure 4.

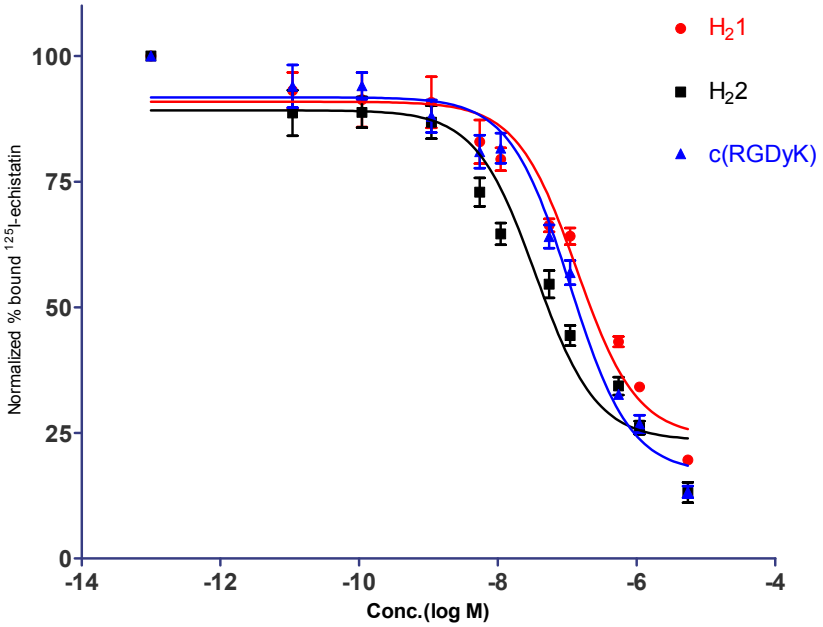


Figure 4. The $\alpha_\upsilon\beta_3$ binding affinities of H_2**1** and H_2**2** measured by a competitive cell-binding assay using U87MG cells where ^{125}I-echistation was employed as $\alpha_\upsilon\beta_3$-specific radioligand. The IC_{50} values of c(RGDyK), H_2**1,** and H_2**2**, were determined to be 110, 139 and 35 nM, respectively (n = 5).

The multiple-step synthetic route outlined in Schemes 1 – 3 involves three parts: (a) synthesis of orthogonally protected compounds **5** and **7;** (b) formation of NHS-activated ester intermediates **6** and **9** after selective deprotection of the peripheral carboxylate groups; and (c) conjugation of c(RGDyK) to the NHS esters followed by acid deprotection of the inner carboxylate groups to form the products: H_2**1** and H_2**2**. The alkylation of CB-cyclam by α-bromoglutaric acid-1-tertbutyl ester-5-benzyl ester **3** was asynchronous at the two non-bridged nitrogen atoms, in which the monoalkylation product **4** predominates at room temperature with 35 – 55 % yield even at the presence of excess of **3** (*10*). In contrary, the dialkylation homologous product **7** could only be obtained at an elevated temperature of 50 °C using two equivalents of **3** to CB-cyclam with 20-45% yield and was always accompanied with the monoalkylation product. Although **7** is hard to be eluted by most of organic solvent on silica gel, a good separation was obtained by adding 5-10% of isopropyl amine in ethyl acetate and **7** was easily purifed on silica gel column. It is noteworthy that the debenzylation of the peripheral carboxylate groups catalyzed by 10% Pd/C in a hydrogen atmosphere always leads to the formation of corresponding esters if the reaction is carried out in an alcohol solvent (e.g. methanol, ethanol). This is likely due to the "proton sponge" nature of the CB-cyclam core that induces the transestification during the process of debenzylation. No clean debenzylization could be accomplished in either methanol or ethanol even in the presence of formic acid as described in literature (*10*). Our method using a mixture of THF/H_2O (see the supporting materials) successful circumvented this problem and afforded debenzylation products in quantitative yield, which were activated by NHS (N-Hydroxysuccinimide) and then conjugated to RGD at the presence of 10 equivalent of N,N-diisopropylethylamine (DIPEA). After HPLC purification, H_2**1** and H_2**2** were obtained by remove -tBu using 95% of TFA.

Both H_2**1** and H_2**2** were efficiently labeled by ^{64}Cu at 70 °C in 0.4 M NH_4OAc buffer within 30 min. The specific activity of ^{64}Cu-**1** and ^{64}Cu-**2** was in the range of 8.4 – 20.4 GBq/μmol. The in vitro stability of the ^{64}Cu labeled peptide conjugates was evaluated in rat serum by radio-HPLC. Chromatographic results showed no ^{64}Cu release from the conjugates through the study (out to 48 h). This high stability is rendered by the CB-TE2A moiety in the conjugates. The $\alpha_\upsilon\beta_3$ binding affinities of H_2**1** and H_2**2** were measured by a competitive cell-binding assay using U87MG cells where ^{125}I-echistation was employed as $\alpha_\upsilon\beta_3$-specific radioligand for the competitive displacement (refs). The IC_{50} values of c(RGDyK), H_2**1,** and H_2**2**, which represent their concentrations required to displace 50% of the ^{125}I-echistation bound on the U87MG cells, were determined to be 110, 139 and 35 nM, respectively (n = 5). The slightly decreased $\alpha_\upsilon\beta_3$ binding of H_2**1** as compared to c(RGDyK) indicates a minute impact of CB-TE2A on the binding of c(RGDyK) to the $\alpha_\upsilon\beta_3$ integrin. As anticipated, H_2**2** indeed exhibited a strong divalent effect measured by the multivalent enhancement ratio (MVE) calculated by dividing the IC_{50} value of H_2**1** by that of H_2**2 (**MVE of H_2**2 (**vs H_2**1):** 4**)(*11*)**. The distance between the two RGD motifs in H_2**2** is maintained greater than 25 bonds (including the arm of lysine), the minimum spacing length required to realize multivalent binding of RGD motifs to the $\alpha_\upsilon\beta_3$ integrin (*12*).

Key Research Accomplishments

1. The inhibitive effects of various Glycosaminoglycans (GAGs) on the uptake of FITC-R9, FITC-R11 and FITC-R13 have been evaluated in four prostate cancer cell lines. The results show that dextran sulfate, protamin sulfate and pentosan sulfate are more potent than heparan sulfate and chondroitin sulfates to inhibit FITC-R11 uptake.
2. In vitro and in vivo evaluation experiments of FITC-R9, FITC-R11 and FITC-R13 have been performed. Of the peptides evaluated, FITC-R11 exhibited the highest uptake in all the four prostate cell lines, the prostate tissue, and PC-3 tumor, indicating the efficiency of uptake was specific to the peptide length.
3. The key multifunctional chelators, CB-TE2A-(COOH) and CB-TE2A-$(COOH)_2$, have been successfully synthesized and used to build multivalent peptide constructs. Preliminary in vitro binding assay clearly showed the desired multivalent effect rendered by the designed scaffold. Standard radiochemical procedures have been established to label the peptide conjugates with ^{64}Cu efficiently. Radiolabeled conjugates showed high in vitro and in vivo stability.

Reportable Outcomes

One manuscript describing the synthesis of the multivalent chelators and the preliminary evaluation of their peptide conjugates has been submitted to ***Journal of the American Chemical Society***.

Conclusions

Glycosaminoglycans play an important role in the uptake of FITC-R9, FITC-R11 and FITC-R13 in four prostate cancer cell lines. Among the inhibitors tested, dextran sulfate, protamin sulfate and pentosan sulfate are more potent than heparan sulfate and chondroitin sulfates. FITC-R11 exhibited the highest uptake in all the four prostate cell lines, the prostate tissue, and PC-3 tumor, indicating the efficiency of uptake was specific to the peptide length. The synthesis of the proposed multifunctional chelators has been successfully accomplished and their multivalent peptide constructs have been obtained. The peptide constructs were efficiently labeled with ^{64}Cu under mild conditions with 30-min. Preliminary in vitro binding assay demonstrated the desired multivalent effect rendered by the designed scaffold, and the radiolabeled conjugates showed high in vitro and in vivo stability.

References:

(1) Nakase, I., Takeuchi, T., Tanaka, G., and Futaki, S. (2008) Methodological and cellular aspects that govern the internalization mechanisms of arginine-rich cell-penetrating peptides. *Adv Drug Deliver Rev 60*, 598-607.

(2) Kinyanjui, M. W., and Fixman, E. D. (2008) Cell-penetrating peptides and proteins: new inhibitors of allergic airways disease. *Can J Physiol Pharmacol 86*, 1-7.

(3) Takayama, K., Tadokoro, A., Pujals, S., Nakase, I., Giralt, E., and Futaki, S. (2009) Novel system to achieve one-pot modification of cargo molecules with oligoarginine vectors for intracellular delivery. *Bioconjug Chem 20*, 249-57.

(4) Takeuchi, T., Kosuge, M., Tadokoro, A., Sugiura, Y., Nishi, M., Kawata, M., Sakai, N., Matile, S., and Futaki, S. (2006) Direct and rapid cytosolic delivery using cell-penetrating peptides mediated by pyrenebutyrate. *ACS Chem Biol 1*, 299-303.

(5) Zakutskii, A. N., Chalisova, N. I., and Subbotina, T. F. (2008) [Functional arginine-containing amino acid sequences in peptides and proteins]. *Bioorg Khim 34*, 149-59.

(6) Noguchi, H., and Matsumoto, S. (2006) Protein transduction technology: a novel therapeutic perspective. *Acta Med Okayama 60*, 1-11.

(7) Zhou, J., Fan, J., and Hsieh, J. T. (2006) Inhibition of mitogen-elicited signal transduction and growth in prostate cancer with a small peptide derived from the functional domain of DOC-2/DAB2 delivered by a unique vehicle. *Cancer Res 66*, 8954-8.

(8) Cai, W., Zhang, X., Wu, Y., and Chen, X. (2006) A thiol-reactive 18F-labeling agent, N-[2-(4-18F-fluorobenzamido)ethyl]maleimide, and synthesis of RGD peptide-based tracer for PET imaging of alpha v beta 3 integrin expression. *J Nucl Med 47*, 1172-80.

(9) Wu, Y., Zhang, X., Xiong, Z., Cheng, Z., Fisher, D. R., Liu, S., Gambhir, S. S., and Chen, X. (2005) microPET imaging of glioma integrin {alpha}v{beta}3 expression using (64)Cu-labeled tetrameric RGD peptide. *J Nucl Med 46*, 1707-18.

(10) Boswell, C. A., Regino, C. A., Baidoo, K. E., Wong, K. J., Bumb, A., Xu, H., Milenic, D. E., Kelley, J. A., Lai, C. C., and Brechbiel, M. W. (2008) Synthesis of a cross-bridged cyclam derivative for peptide conjugation and 64Cu radiolabeling. *Bioconjug Chem 19*, 1476-84.

(11) Montet, X., Funovics, M., Montet-Abou, K., Weissleder, R., and Josephson, L. (2006) Multivalent effects of RGD peptides obtained by nanoparticle display. *J Med Chem 49*, 6087-93.

(12) Li, Z. B., Cai, W., Cao, Q., Chen, K., Wu, Z., He, L., and Chen, X. (2007) (64)Cu-labeled tetrameric and octameric RGD peptides for small-animal PET of tumor alpha(v)beta(3) integrin expression. *J Nucl Med 48*, 1162-71.